

## TECHNICAL NOTE

Margaret C. Kline,<sup>1</sup> M.S.; David L. Duewer,<sup>2</sup> Ph.D.; Janette W. Redman<sup>1</sup>;  
and John M. Butler,<sup>1</sup> Ph.D.

# Results from the NIST 2004 DNA Quantitation Study\*

**ABSTRACT:** For optimal DNA short tandem repeat (STR) typing results, the DNA concentration ([DNA]) of the sample must be accurately determined prior to the polymerase chain reaction (PCR) amplification step in the typing process. In early 2004, the National Institute of Standards and Technology (NIST) conducted an interlaboratory study to help assess the accuracy of DNA quantitation in forensic DNA laboratories. This study was designed with four primary purposes: (1) to examine concentration effects and to probe performance at the lower DNA concentration levels that are frequently seen in forensic casework; (2) to examine consistency with various methodologies across multiple laboratories; (3) to examine single versus multiple source samples; and (4) to study DNA stability over time and through shipping in two types of storage tubes. Eight DNA samples of [DNA] from 0.05 ng/ $\mu$ L to 1.5 ng/ $\mu$ L were distributed. A total of 287 independent data sets were returned from 80 participants. Results were reported for 19 different DNA quantitation methodologies. Approximately 65% of the data were obtained using traditional slot blot hybridization methods; 21% were obtained using newly available quantitative real-time PCR (Q-PCR) techniques. Information from this interlaboratory study is guiding development of a future NIST Standard Reference Material for Human DNA Quantitation, SRM 2372.

**KEYWORDS:** forensic science, DNA typing, DNA quantitation, interlaboratory study, quantitative real-time PCR (Q-PCR)

The determination of the quantity of human DNA present in a sample prior to amplification with the polymerase chain reaction (PCR) is an important step in forensic DNA analysis using multiple short tandem repeat (STR) markers. There are both regulatory and measurement performance reasons for this importance. The U.S. Federal Bureau of Investigation's standards, which govern forensic DNA typing in the United States, require that a laboratory shall have and follow a procedure for evaluating the quantity of the human DNA in the sample when possible (1). More germanely, the best performance with multiplex PCR amplification of STR markers using commercially available kits occurs within a fairly narrow range of input DNA amount—typically on the order of 0.5 ng to 2.5 ng (2–5). Too much DNA can lead to off-scale fluorescent signal and a variety of PCR artifacts, including: imbalance in the multiplex PCR amplification, incomplete adenylation of PCR products,

and enhanced strand-slippage or “stutter” of various forms (6,7). Too little DNA can result in stochastic amplification that causes imbalance within and between loci and even allele dropout (8,9).

Over the past decade, our group at NIST has been involved with a series of interlaboratory studies to help forensic DNA typing laboratories assess their performance with various technologies and issues relevant to forensic testing (10–17). The most recent of these studies indicated that the accuracy of DNA quantitation does impact the quality of STR typing, particularly when examining mixture samples (16,17). In order to better understand the measurement performance of different DNA quantitation techniques, we initiated the NIST 2004 DNA Quantitation Study (QS04). Results from this study enable evaluation of the relative precision and bias of current DNA quantitation methods at low ng/ $\mu$ L levels. QS04 also provided information on the stability of DNA samples under standard storage and shipping conditions that is guiding the development of a human DNA quantitation Standard Reference Material, SRM 2372.

<sup>1</sup> Biotechnology Division, National Institute of Standards and Technology, Gaithersburg, MD 20899.

<sup>2</sup> Analytical Chemistry Division, National Institute of Standards and Technology, Gaithersburg, MD 20899.

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## Materials and Methods

### DNA Samples

The QS04 sample materials consisted of eight extracted DNA samples, 100  $\mu$ L each, in 10 mmol/L Tris, 0.1 mmol/L EDTA buffer (TE<sup>-4</sup>). The samples were labeled “A” through “H”. Samples A, B, C, and D were dilutions of a pooled lyophilized human DNA product D 7011 (Sigma, St. Louis, MO). A stock solution was made by reconstituting 5.06 mg of the lyophilized DNA in 50 mL of TE<sup>-4</sup> and allowing it to equilibrate for 11 weeks in a Teflon container at 4°C. The final DNA concentration ([DNA]) of the stock was determined by UV absorbance at 260 nm on a Cary 100



TABLE 1—Quantitation Methods Used in QS04. Key for detection and instrument codes is listed below. There are 34 categories and 19 unique “codes” or quantitation methods examined.

Class	Method	Principle	Signal	Instrument	Code	Labs	Set	Ref
Direct, non-blot	AluQuant	Luminescence	Probe	TLA	a	4	9	23
"	"	"	"	TD20/20	a	3	3	"
"	"	"	"	TR	a	1	1	"
"	Picogreen	Fluorescence	Picogreen	FA	p	2	3	24
"	"	"	"	LF320	p	1	1	"
"	"	"	"	PBC4000	p	1	1	"
"	"	"	"	PK_FL	p	1	2	"
"	"	"	"	SMAX	p	2	3	"
"	"	"	"	?	p	1	2	"
"	Yield Gel	"	EtBr	visual	y	3	14	18
Direct, blot	“ACES”	Luminescence	Solulink	visual	A	4	10	21
"	"	"	"	KI	A	1	4	"
"	QuantiBlot	"	ECL	visual	E	18	40	20
"	"	"	"	CCDBIO	E	2	11	"
"	"	"	"	KI	E	3	7	"
"	"	"	SSWD	visual	D	3	11	"
"	"	"	SSWF	visual	F	4	6	"
"	"	Colorimetry	TMB	visual	T	31	98	"
Endpoint PCR	BodeQuant	Fluorescence	Picogreen	CF4000	—	1	1	32
Real Time PCR	Quantifiler	Fluorescence	Probe	ABI7000	0	16	32	25
"	"	"	"	ABI7700	0	1	2	"
"	"	"	"	ABI7900	0	2	3	"
"	Alu Q-PCR	"	Sybr	RG3000	1	2	4	26
"	"	"	"	ABI7000	1	1	1	"
"	Alu_Sifis	"	"	i-Cycler	2	1	2	27
"	Alu_tqman	"	Probe	ABI7000	3	1	1	—
"	Aluprobe	"	"	ABI7700	4	1	1	—
"	BRCA 1	"	"	ABI7700	5	1	2	28
"	"	"	"	ABI7900	5	1	1	"
"	CFS-HUMRT	"	"	ABI7000	6	1	1	29
"	"	"	"	ABI7900	6	1	3	"
"	GB:L78833.1	"	"	i-Cycler	7	1	2	—
"	RB1	"	"	ABI7700	8	1	3	30
"	RTALU	"	"	ABI7000	9	1	2	31
						118	287	

**Detection Codes**

- ECL Enhanced Chemiluminescence (Amersham, Arlington Heights, IL)
- EtBr Ethidium bromide
- Picogreen Picogreen Reagent (Molecular Probes, Eugene, OR)
- SSWD Super Signal West Dura (Pierce Biotechnology Inc, Rockford, IL)
- Sybr SYBR Green I Reagent (Molecular Probes, Eugene, OR)
- TMB Chromgen:TMB 3,3',5,5'- Tetramethylbenzidine (Applied Biosystems, Foster City, CA)
- Solulink ACES probes (Solulink Inc, San Diego, CA)
- SSWF Super Signal West Femto (Pierce Biotechnology Inc, Rockford, IL)

**Instrument Codes**

- ABI7000 ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA)
- ABI7700 ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA)
- ABI7900 ABI Prism 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA)
- CCDBIO Hitachi CCD Bio Imager, (MiraiBio Inc., Alameda, CA)
- CF4000 CytoFluor 4000 Multiwell Fluorescent Plate Reader (Applied Biosystems, Foster City, CA)
- FA Fluoroskan Ascent (Thermo Electron Corp., Waltham, MA)
- i-Cycler iCycler Opitcal System (BIORAD, Hercules, CA)
- KI Kodak Imager 440, (Kodak, Rochester, NY)
- LF320 Lambda Fluoro 320 Plus (MWG Biotech, Highpoint, NC)
- PBC4000 Perspective Biosystems Cytofluor 4000 (Perspective Biosystems, Framington, MA)
- PK\_FL Packard FluoroCount (Packard Instrument Co. Inc., Meriden, CT)
- RG3000 Corbett Research Rotorgene 3000 (Phoenix Research, Hayward, CA)
- SMAX SpectraMax Gemini Spectrofluorometer (Molecular Devices Corp., Sunnyvale, CA)
- TD20/20 Turner TD-20/20 Luminometer (Turner BioSystems, Inc, Sunnyvale, CA)
- TLA Thermo Luminoskan Ascent (Thermo Electron Corp., Waltham, MA)
- TR Turner Reporter Microplate Luminometer (Turner BioSystems, Inc, Sunnyvale, CA)

summarize these data. We have arbitrarily assigned these data to have quantitative values of twice the reported limit.

The remaining 160 semi-quantitative data were reported as upper limits (e.g., <0.031 ng/μL), with the majority of the limits at the lowest dilution of the calibration series in common use with

the given method. More than 75% of the fully quantitative values for the given samples were greater than the specified maximum values. For these 123 data, assignment of any value less than the limit has little influence on robust statistical summaries. We have arbitrarily assigned these data to have quantitative values of



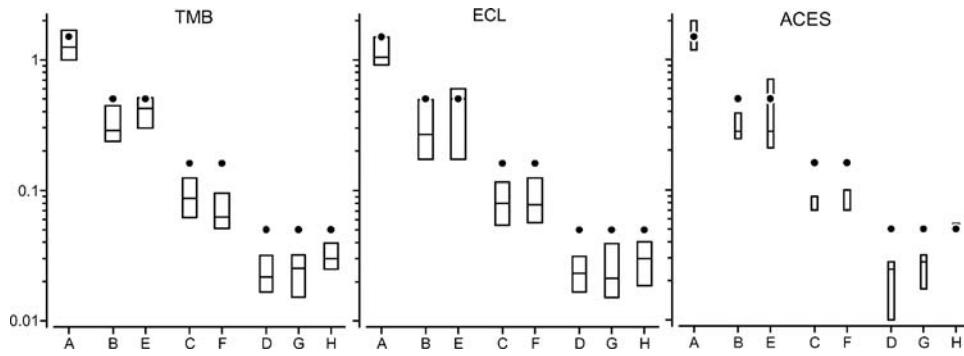


FIG. 2—Box plot summary of the distribution of among-laboratory results for the major subgroups of the blot-based methods: TMB, ECL, and “ACES.” The subgraph design is described in Fig. 1.

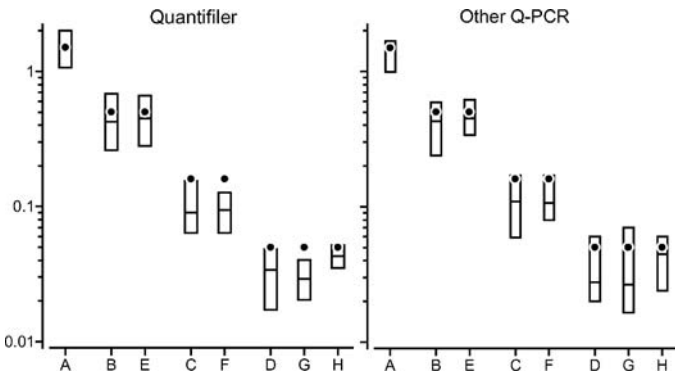


FIG. 3—Box plot summary of the distribution of among-laboratory results for the Quantifiler method and for the aggregate of all other Q-PCR-based methods. The subgraph design is described in Fig. 1.

TABLE 3—Consensus [DNA] and estimates of variability.

Sample	[DNA], ng/μL			Multiplicative Standard Deviations			
	Target	Median*	% Recovery†	S <sub>rep</sub> ‡	S <sub>dup</sub> §	S <sub>among</sub>	S <sub>among</sub> ¶
A	1.50	1.25	83	1.3	1.5	1.5	—
B	0.50	0.32	64	1.5	1.6	1.9	1.4
E	0.50	0.42	84	1.4	1.6	1.8	1.6
C	0.16	0.090	56	1.6	1.6	1.7	1.4
F	0.16	0.084	53	1.6	1.5	1.8	1.5
D	0.050	0.028	56	1.5	1.5	1.9	1.6
G	0.050	0.028	56	1.5	1.6	1.8	1.6
H	0.050	0.037	73	1.8	1.5	1.6	1.7
Average			66	1.5	1.5	1.8	1.5

\* Median of the quantitative among-laboratory results for all methods, where the number of results varies from 103 to 117 depending on sample.

†  $100 \times (\text{Median [DNA]})/(\text{Target [DNA]})$ .

‡ Pooled multiplicative standard deviation of within-analyst replicates for all methods, where the number of replicate assays varies from 15 to 22 depending on sample.

§ Pooled multiplicative standard deviation of within-laboratory duplicates for all methods, where the number of duplicate assays varies from 52 to 64 depending on sample.

|| Robust multiplicative standard deviation of the among-laboratory results for all methods, where the number of results varies from 103 to 117 depending on sample.

¶ Robust multiplicative standard deviation of the among-laboratory results for all methods after standardization to the value reported for sample A.

combined and for the four major exclusive groups: all blot-based results, all Q-PCR results, and the two non-blot methods AluQuant and Picogreen. The consensus [DNA] for each set of results for each sample is indicated by the location of the median line within each box, the variability of the central 50% of results is indicated by the length of the box along the vertical axis, and the relative number of results is indicated by the width of the box along the horizontal axis. The pattern of results among the eight samples is similar for all methods, with the exception that the Picogreen-based methods over-estimate the [DNA] of the 0.05 ng/μL samples D, G, and H relative to the other assays. It is probable that this results from imperfect correction of background fluorescence with this assay at such low [DNA]; observation of a signal does not ensure that the signal has quantitative utility.

Figure 2 similarly summarizes the among-laboratory results for the three subgroups of blot-based direct methods: Quantiblot with TMB and ECL detection and the in-house “ACES”-based methods. Again, the pattern of results is quite similar, with the possible exception of the “ACES”-based methods with the Teflon-stored sample H. Figure 3 contrasts the among-laboratory results for Quantifiler with the collection of all other Q-PCR methods. The pattern of results is similar, although the variability of the collection is somewhat greater for the 0.05 ng/μL samples D, G, and H.

Table 3 lists the median [DNA] for the combined set of among-laboratory results. The median is the most commonly used robust estimate of consensus values for interlaboratory data, given the almost inevitable presence in such studies of a small number of “outlier” data (35). The percent recovery of the observed median

relative to the target [DNA] range from 53% to 83% over the eight samples, for an average recovery of 66%. Potential causes of these systematically lower-than-expected consensus values and the effects of differences in DNA source, target [DNA], and the nature of the container are discussed below.

Measurement Variability

Given that the result distributions are quite similar for all adequately sensitive quantitative methods, the variability of current DNA quantitation methods can be estimated from the total set of interlaboratory data without regard for specific method. As noted in prior studies (16,17) and confirmed here by the approximate symmetry of the box-plot distributions when displayed on logarithmic axes, the reported [DNA] are lognormally distributed. The variability for such data is more readily understood when expressed in the units of measurement as multiplicative standard deviations,  $\times\text{SD}$ , where the notation “ $\times$ ” is employed as the multiplicative





